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# A NEW NONLINEAR REGRESSION APPROACH THAT ALLOWS DETECTION OF INTER-INDIVIDUAL DIFFERENCES IN SINGLE-POINT RADIOLIGAND BINDING STUDIES

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In biochemical experiments measuring radioligand binding or enzyme activities, it is often convenient to pool tissue from different animals if only small amounts are available. Pooling of tissues from different animals may conceal or obscure significant and important differences between individuals in a population. Although linear in nature, the statistical approach and solution to test for inter-individual differences is not straightforward. We therefore introduce a new solution with a nonstandard linear regression equation based on nonlinear iterative least squares regression theory. This procedure has the advantage that it is easy to implement and allows for a full statistical exploration of this and even more complex models.

As an example, single-point radioligand binding experiments were carried out to test for inter-individual differences in corticotropin-releasing hormone (CRH) receptor densities in 6 different brain sections of 7 rats. These rats were shown to differ substantially in their behavioral response to central administration of corticotropin-releasing hormone. The studies were performed with [$^{125}$I]Tyr$^{0}$-oCRH as a radioligand, and replicates of total binding and nonspecific binding were determined. The variable of interest (specific binding) was then calculated as the difference between total binding and nonspecific binding.

Two nested regression models were compared using a partial F-test, which in the case of a significant result ($\alpha = 0.05$, two-sided) was followed post-hoc by a modification of Scheffe's test. Using Monte-Carlo randomization techniques, statistical power was empirically determined for each brain section. As a result, only the piriformis cortex was found to have statistically significant inter-individual differences ($p = 0.024$), which is also confirmed by the relatively high power of 82% ($\alpha = 0.05$, two-sided).

Further post-hoc analysis, however, could not resolve the overall significant finding into individual components.This new statistical approach emphasizes the importance of a carefully planned analysis in order to detect inter-individual differences in biochemical and related experiments when the amount of tissue is the constraining factor in the experimental design.

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## INTRODUCTION

There are situations in biochemical research in which a standard multi-point equilibrium or kinetic assay cannot be applied, e.g., enzyme activity measurements or radioligand binding studies. This is particularly true in cases where there is only a small amount of tissue available. To overcome this problem it is convenient to pool regions from different animals. However, this should be done only after one has determined that there are no significant differences among the animals in the study. Any differences that are more than random can seriously bias the overall statistical analysis, unless the investigator is aware of the individual variation and adjusts for it. As will be shown, this statistical analysis involves an extension of standard methodology. Apart from being a confounding variable in experiments, interest in understanding the physiological and biochemical mechanisms that set apart individual members of a species has been growing because analytical tools become are increasingly sensitive in detecting subtle variance between individuals.

As an example for radioligand binding studies, we introduce a new approach using an equation that maintains the simple relationship between the *measured* quantities (total binding and nonspecific binding) and the *derived* variable of interest, the specific binding (SB). The quantity SB is defined as the difference between total binding (TB) and nonspecific binding (NB). Total binding and NB are usually measured with several replicates. However, there is no match between a particular TB replicate and a particular NB replicate. In fact the number of replicates of the two quantities may be different. This poses no problem in defining SB because only the mean values of TB and NB replicates are used for a given animal i,

$$[1] \qquad SB_i = \overline{TB_i} - \overline{NB_i} \quad (i = 1,\ldots,n)$$

An analysis of variance (ANOVA) could not be applied to test the null-hypothesis that there are no statistically significant differences between SB in brain sections of individual animals. To reemphasize, the original data do not have matched pairs of TB and NB; therefore, within-group variability cannot be determined, since there is only a single value of SB for each animal. The authors suspect that no simple modification of ANOVA is appropriate for this situation. Indeed, here it is necessary to formulate this problem as a multiple linear regression model expressed with the aid of a design matrix. This approach, although possible, is cumbersome and therefore we decided to explore designs beyond the general linear model.

We found a solution for that particular problem using a regression equation with 2 dependent variables (TB, NB) and an indicator or index variable, which identifies the parameters to be matched to the observations. There is one such equation for each animal. Although linear in its parameters the unusual nature of this regression equation requires computational flexibility not found in standard regression software packages. However, our implementation of a nonlinear regression program can successfully be applied to this and even more complex systems. Nonlinear regressions include as a special case multiple linear regressions. They are more complex in mathematical theory and have a correspondingly more complex computer implementation, but they are extremely versatile.

CRH, a 41 amino-acid peptide, is widely distributed in the brain and is thought to be the major coordinator in behavioral, neuroendocrine, autonomic, and immunological responses to stress (1). Our primary interest was the detection of significant inter-individual differences in CRF binding in a relatively small population of rats that had previously been shown to differ in their behavioral response to centrally administered CRF.

## BINDING METHODS

**Animals**. Adult male Long-Evans Hooded rats weighing 325-340 g were used throughout this study. Animals were housed in a light- and temperature-controlled room, with controlled administration of food and water. Stainless-steel guide cannulae were implanted with access to the lateral ventricle for the i.c.v. administration of oCRH (2). The rats were allowed to recover for 1 week before they received weekly i.c.v.-injections of increasing CRH doses (0.1 to 10 μg) over a time period of 2-3 months to determine the CRH dose-response curve for each animal.

**Materials**. Iodinated ovine CRH, [$^{125}$I]Tyr$^{0}$-oCRH ($^{125}$I-oCRH), with a specific activity of 2200 Ci/mmol was obtained from New England Nuclear (Boston, MA), and unlabeled ovine CRH (oCRH) and unlabeled rat/human CRH (r/hCRH) were purchased from Bachem (California). All other standard reagents were purchased from Sigma (St. Louis, MO).

**Tissue preparation**. Rats were sacrified by decapitation, and brain regions of interest were dissected, weighed, and placed in 20 volumes of ice-cold buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.2).

**[$^{125}$I]Tyr$^{0}$-oCRH binding assay**. The CRH receptor binding assay was conducted essentially as described elsewhere (3). Briefly, tissues of various brain regions were homogenized in 4 ml of buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.2) using a Brinkman polytron (setting 6 for 10 s). The homogenate was first centrifuged at 30,000 x g for 10 min at 4 °C; the resulting pellet was discarded and this process was repeated a second time. The pellets were resuspended in an incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM

EGTA, 0.1 % BSA, aprotinin (100 kallikrein units/ml) and 0.1 mM bacitracin, pH 7.2) to a final protein concentration of 0.1 - 1.2 mg/ml assay. The final protein concentration of each membrane preparation was determined using bovine serum albumin (BSA) as a standard (4).

**Incubation assay:** 100 µl of the membrane suspension, 100 µl of incubation buffer containing $^{125}$I-oCRH (final concentration 0.1 nM), and 50 µl of incubation buffer containing oCRH (5.4 nM final concentration) was added to 1.5 ml polypropylene microcentrifuge tubes and incubated in quadruplicate with 50 µl of the incubation buffer or incubated in triplicate with 50 µl of incubation buffer containing unlabeled r/hCRH (1 µM r/hCRH) to define nonspecific binding. All binding incubations were initiated by the addition of membrane protein. The reaction was allowed to proceed for 2 h at 22 °C. The tissues were separated from the incubation medium by centrifugation in a microcentrifuge for 3 min at 12,000 x g. The resulting pellets were washed gently with 1 ml of ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 2.6 mM KCl, 10 mM $Na_2HPO_4$, 1.76 mM $KH_2PO_4$), pH 7.4, containing 0.01 % Triton X 100. Then the contents were recentrifuged for 3 min at 12,000 x g; the supernatent was aspired and the radioactivity of the pellets were measured in a gamma counter at 80% efficiency.

Single-point CRH-receptor binding was performed using an approximate receptor-saturating concentration of 0.1 nM [$^{125}$I]-Tyr$^{0}$-oCRH plus 5.4 nM unlabeled oCRH to determine TB. Nonspecific binding was defined as the binding not displaced by 1 µM r/hCRH. The total concentration of ligand used consisted of the concentrations of radiolabeled and unlabeled ligand, [L*] and [L], respectively. To account for the dilution with unlabeled ligand, SB was divided by the fraction of labeled ligand f: $f = [L^*]/([L^*] + [L])$.

The standard error of specific binding ($SEM_{SB}$) represented in the figures was calculated as the difference between the means of two groups with unequal number of data points (TB with 4 replicates and NB with 3 replicates). Equation 2 is expressed in terms of the two samples standard-deviations ($SD_{TB}$ and $SD_{NB}$) and the two sample sizes ($n_{TB}$ and $n_{NB}$).

$$[2] \qquad SEM_{SB} = SD_{pooled} \cdot \sqrt{\frac{1}{n_{TB}} + \frac{1}{n_{NB}}}$$

$$\text{where} \quad SD_{pooled} = \sqrt{\frac{(n_{TB} - 1) \cdot SD_{TB}^2 + (n_{NB} - 1) \cdot SD_{NB}^2}{n_{TB} + n_{NB} - 2}}$$

## DATA ANALYSIS

Our goal is to test the null-hypothesis of no inter-individual differences in specific binding (SB) of a given tissue. To accomplish this we have formulated two nested models to which we apply nonlinear regression methods. We chose iterative nonlinear least-squares regression techniques (algorithm according to Marquardt (5)). For statistical comparison of the two models a partial F-test was performed that used certain output-quantities of the regression analysis. A modified Scheffe-test was applied to resolve a significant overall result ($\alpha = 0.05$, two-sided) post-hoc into individual components. Statistical power of the experimental design was established using Monte-Carlo randomization techniques.

In general, our regression method minimizes the NORM, which is defined as the weighted sum of squared error (SSE) as in Eq. 3.

$$[3] \qquad NORM \equiv SSE(\mathbf{B}) = \sum_{i=1}^{n} \frac{[y_i - f(\mathbf{B}, x_i)]^2}{\sigma_i^2}$$

$y_i$ ($i = 1, \ldots, n$) are the values of the dependent variable, n = number of datapoints, f describes a general mathematical relation between dependent (y) and independent (x) variables, **B** is the parameter-vector to be optimized, $x_i$ are the values of the independent variable x and $\sigma_i$ is the standard-deviation of the dependent variable at a datapoint ($x_i$, $y_i$). A non-weighted scheme was used and the individual standard-deviations ($\sigma_i$) were set to 1. An important note: for the regression equation, $y_i$ represents $TB_i$ for some of the data and $NB_I$ for other data.

We selected the sum of squared error as a "goodness-of-fit" criterion. In comparing equations with different numbers of parameters, the curve generated by the more complicated equation (the one with more parameters) will generally come closer to the data points. The question is whether this decrease in sum of squared error is worth the "cost" of additional parameters (loss of degrees of freedom). When comparing hierarchical models, the probability that additional parameters are without effect on the sum of squared error is defined by an F-distribution (6). The F-ratio quantifies the relationship between the relative increase in sum of squared error and the relative increase in degrees of freedom and is calculated according to Eq. 4.

$$[4] \qquad F = \left[ \frac{\frac{(SSE_1 - SSE_2)}{(df_1 - df_2)}}{\frac{SSE_2}{df_2}} \right]$$

where $SSE_i$ is the sum of squared error and $df_i$ is the degree of freedom (for model 1 and 2, i = 1,2). The two values of $SSE_i$ and $df_i$ are obtained from two analyses to the same data set,

differing only in the number of parameters. In the reduced model some of the parameters of the full model are fixed at zero. If the reduced model is correct, the expected F-ratio is near 1.0. If the ratio is much greater than 1.0, there are two possibilities: (1) the full model is correct or (2) the reduced model is correct, but random scatter led the more complicated model to fit better. The p value expresses how rare this coincidence would be and answers this question: if the reduced model (Index 1) is really correct, what is the chance to randomly obtain data that fits the full model (Index 2) significantly better? If the p-value is low, one concludes that the full model fits the data significantly better than the reduced model. Levels of significance corresponding to the calculated value of F can be computed from the probability function of the F-distribution, which has ($df_1$ - $df_2$) degrees of freedom for the numerator and $df_2$ for the denominator. To compute the p-value the C-subroutine betai was implemented (7).

In the present analysis two non-standard regression models that are linear in the parameters were defined. One of them can be expressed as a restricted case of the more general form (nested design). These models assume that variances of TB and NB are homogeneous across all animals. Both models were fit to the original data set, including TB and NB points being corrected for differences in protein concentrations. The restricted simple model consists of M+1 parameters to be estimated, one of these representing NB for each of the M animals and one additional parameter for the common value SB for all animals. The general model consisted of 2·M parameters to be estimated, one NB parameter and one SB parameter for each of the M animals. To express the general model in a regression form we rearranged Eqs. 2 - 5. Note that the vector **B** can represent either of two types of observed binding and thus Eq. 5 is of the multiple dependent variable type regression described above.

$$[5] \qquad \mathbf{B}[i] = \begin{cases} \mathbf{NB}[i] + k \cdot SB & \text{for Model 1} \\ \mathbf{NB}[i] + k \cdot \mathbf{SB}[i] & \text{for Model 2} \end{cases}$$

$$\text{where } k = \begin{cases} 0 & \text{if datapoint represents nonspecific binding} \\ 1 & \text{if datapoint represents total binding} \end{cases}$$

where the index i refers to the i-th animal (i = 1,...,7). When a datapoint is a nonspecific binding measure, k is set to zero and the dependent variable **B** represents nonspecific binding. If the datapoint is a measure of total binding then k is set to 1 and **B** represents total binding. **NB** and **SB** are the parameter vectors to be simultaneously optimized.

A full description of the nonlinear regression method may be found elsewhere (8). Briefly, the first step is input of data and the starting values for the parameters to be estimated followed by calculation of the model dependent variable **B**, as outlined above (Eq. 5). Then the difference between the measured datapoints (TB, NB) and the calculated function **B** of the model has to be squared and summed over all data points (Eq. 3). The resulting value is called the sum of squared error (SSE). In order to find the optimal parameters for the model function, SSE has to become a minimum. At that minimum, all partial derivatives from SSE with respect to each parameter becomes zero. Those numerical calculated partial derivatives are used with further operations to select a new set of parameters. Again, the SSE is found by taking the sum over the squared difference between the measured point (TB, NB) and the function **B**. All this is repeated

until relative changes in the parameter estimates and successive changes in SSE are less than a certain tolerance (here $10^{-6}$), at which point convergence and a final solution is declared.

The approximate standard error $SE_k$ on each parameter can be calculated from the diagonal elements of the covariance matrix COV according to Eq. 6. To calculate $SE_k$, a derived quantity, the estimated variance of the observations (MSE : mean squared error) has to be calculated after the optimization. $SSE_{min}$ is the weighted sum of squared error (Eq. 3) at the minimum and the difference between the number of measurements and the number of parameters is the degrees of freedom (df). The resulting formula is stated in Eq. 6.

$$[6] \qquad SE_k = \sqrt{cov_{kk} \cdot MSE} \qquad \text{where} \quad MSE = \frac{SSE_{min}}{df}$$

The parameter coefficient of variation in percent (%CV) was calculated according to the ratio of the parameter standard-deviation $SE_k$ and the parameter estimate $b_k$ times 100.

$$[7] \qquad \%CV_k = 100 \cdot \frac{SE_k}{b_k}$$

Correlations in optimization procedures arise when two or more parameters try to explain the same data. The correlation coefficient between any two parameters ($corr_{kl}$) is given by the scaled, off-diagonal elements of the covariance matrix (Eq. 8). The parameters are mutually independent when $corr_{kl}$ is zero, on the other hand if $corr_{kl}$ is $\pm$ 1, they are explaining the data in the exact same way.

$$[8] \qquad corr_{kl} = \frac{cov_{kl}}{\sqrt{cov_{kk} \cdot cov_{ll}}}$$

Several statistics calculated during the optimization procedure may be used as indicators of "goodness-of-fit." These are the root mean squared error and the coefficient of determination.

The root mean squared error (RMS) measures the average deviation around the fitted curve. RMS in percent is defined in Eq. 9.

$$[9] \qquad \%RMS = 100 \cdot \sqrt{MSE}]$$

The coefficient of determination ($R^2$) is a measure of the fraction of the total variance accounted for by the model and calculated as

$$[10] \qquad R^2 = 1 - \frac{SSE_{min}}{SSY_w}$$

where $SSE_{min}$ is the weighted sum of squared error (Eq. 3) at the minimum and $SSY_w$ is defined as $\Sigma [(y_i - y_{mean})/ \sigma_i]^2$ with $y_{mean}$ as the weighted arithmetic mean of the y-values.

In case the overall F-test revealed a p value lower than the significance level ($\alpha = 0.05$, two-sided), a modified Scheffe-test was performed. Of the approaches considered for controlling Type I error in multiple comparisons, a number of tests are available, but the Scheffe method is the only one that is based on the same assumptions as the overall F-test in the analysis of variance (c.f. Winer et al. (9)). Regarding our model, out of the 14 parameter estimates (means) only 7 estimates associated with SB should be contrasted. The within-group degrees of freedom can be determined as $df_{wg} = n_{tot} - n_{par} = 49 - 14 = 35$, where $n_{tot}$ are the total number of data

points and $n_{par}$ are the number of all parameters to be estimated. The degrees of freedom for the between group variability can be written as $df_{bg} = M - 1 = 7 - 1 = 6$, where M is the number of animals. The F-statistic according to the ideas of Scheffe can be calculated using the SB parameter estimates and their associated variances Var(SB) as calculated from the main diagonal of the variance-covariance matrix.

$$[11] \quad F(df_{bg}, df_{wg}) = \frac{(SB[i] - SB[j])^2}{df_{bg} \cdot (Var(SB[i] + Var(SB[j]))}$$

where $i,j = 1,...,7$ and $i \neq j$. This formula assumes parameters SB[i] and SB[j] are uncorrelated ($corr_{i,j} = 0$) as is the case for our data. Note that the variance of the SB parameter estimates can also be expressed in terms of the mean squared error (cf. Eq. 6) and the number of associated data points: $Var(SB[i]) = MSE \cdot (1/n_{TB} + 1/n_{NB})$. The relationship between the F-distribution and the incomplete $\beta$-function was used to compute a probability p (7).

In Monte-Carlo simulations, random noise with constant error was added to each point calculated on the theoretical mean $B_{true}$ with an expected standard-deviation of RMS (c.f. Table 1). The resulting random datapoint $B_{const}$ can be expressed as

$$[12] \quad B_{const} = B_{true} + RMS \cdot RND$$

$$\text{where } B_{true} = \begin{cases} B_{NB} & \text{if data point is generated as NB} \\ B_{NB} + B_{SB} & \text{if data point is generated as TB} \end{cases}$$

$B_{NB}$ and $B_{SB}$ are the optimized parameters of the full model. RND is a normal random deviate with a mean of zero and a unit variance, N(0,1). Generation of a uniformly distributed random number and conversion into a normal deviate was implemented using the subroutines ran1 and gasdev given in *Numerical Recipes in C* (7).

All calculations were performed by using computer programs written in Turbo-C++ 4.5 (Borland Inc.) to run on a pentium IBM-type Personal Computer under Windows NT.

## RESULTS AND DISCUSSION

Equilibrium single-point radioligand binding experiments were carried out to test the hypothesis of significant inter-individual differences across animals in a given tissue preparation. Six different brain regions were chosen and replicates of TB and NB were determined. One data point of section #1 was considered to be an outlier and was dropped (more than 3 standard-deviations from the mean).

A nonlinear regression approach was chosen to overcome some implementation problems under the general linear model as explained in the section on Data Analysis. Our approach can be formulated and solved in terms of two simple nested regression models (Eq. 5). Another advantage is the use of all original datapoints (TB and NB), corrected only for differences in protein contents.

Starting values for the 8 parameters of Model 1 (7 NB and 1 SB) were taken from the arithmetic means of the NB datapoints (n = 3) and from the arithmetic mean of all SB across the M animals calculated according to Eq. 2 (n = 7). Part of the starting values for the 14 parameters (7 NB and 7 SB) for the subsequent analysis of the same data set with Model 2 were taken from

the optimized parameter values of Model 1. Starting values for Parameters 9 to 14 (all SB) were identical to the starting value of Parameter 8. All regression cycles converged after 5 iterations. Using the SSE and the proper degrees of freedom of both models, an F-ratio according to Eq. 4 was calculated and a p-value under the F-probability distribution determined. In case $p \leq 0.05$, a modified Scheffe-test was performed trying to resolve the overall result post-hoc for individual differences.

Certain summary statistics are presented in Table 1. The SSE in the full model is never larger than in the reduced model. Recall that RMS is the square root taken of SSE divided by the proper degrees of freedom. The degrees of freedom for the full model is always less than that for the reduced model. In general the RMS will be smaller for the full model than the reduced model, only when the extra parameters significantly reduce the SSE (when the full model really fits the data better). As expected, $R^2$ as a descriptive measure of the "goodness-of-fit" is always greater for the full model than for the reduced model. However, the significance of this difference is determined by the overall partial F-Test (refer to Table 2, last column). Only the two $R^2$ of Section #3 (piriformis) reach statistically significant differences at the 0.05 level. The Section #2 is significant at the 0.1 level. All other sections show no significant differences between the $R^2$ of both models.

For each section the reduced model has more precise estimates of its parameters, reflected by its lower $\%CV_{max}$. Even for the full model the estimates were sufficiently precise since $\%CV_{max}$ was always less than 2/3. Recall, that $CV_{max}$ is the maximum value of all the ratios of standard error of the parameter divided by its estimate. As can be predicted from a matrix analysis of the regression equation, the standard deviation for each parameter depends on the

sample standard deviation and number of observations of TB and NB for each animal. Therefore, in case all the animals have the same number of TB ($n_1$) and NB ($n_2$) observations, the parameter standard deviations for all $TB_i$ were the same and likewise for $NB_i$ (results not shown).

In Fig. 1 specific CRH-receptor densities in the hypothalamus, hippocampus, piriformis, locus coeruleus, frontal cortex, and cerebellum of 7 individual rats are compared. Table 1 summarizes some results of the regression analysis of the 2 models. The statistical analysis for model discrimination shown in Table 2 indicates no significant differences between individuals at the 0.01 level. However, at the 0.05 level the piriformis region reveals significant inter-individual differences, and the hippocampus region is significant at the 0.1 level. For the piriformis section, a further post-hoc contrast among all rats was performed using a modified version of the Scheffe-test (c.f. Section Data Analysis). In Table 3 the results for sections piriformis are presented.

Even if important differences are there, one might not obtain statistically significant differences in the experiment. Just by chance, the data may yield a p-value greater than the significance level $\alpha$. When interpreting a result of an experiment that found no significant difference, one must know how much power the study had to find various hypothetical differences, if they existed. The power depends mainly on the sample size and the amount of variation within the groups, as quantified by the standard-deviation. One way to determine power is to analyze many experiments that have the original number of sample size ($n_{tot}$), but different artificial values, and calculate a p value for each experiment. Statistical power is the fraction of these experiments that have a p value less than $\alpha$ and thus are declared statistically significant. Table 2 shows the result of empirically determined power for the 0.05 significance

levels. It is evident that only the data from Section 3 (piriform cortex) have sufficient power to detect a possible true difference in an analysis. However, contrasting the individual means of this brain section do not indicate which is different (Table 3).

In summary, statistical analysis of possible inter-individual differences in SB between rats failed to reject the Null-hypothesis at the 0.01 level. However, in one region (piriformis cortex) the Null-hypothesis could be rejected at the 0.05 level and in another region (hippocampus) at the 0.1 level (see also Table 3). A further multiple comparison of the section piriformis could not be resolved for individuals. Results of a statistical power-analysis showed that a high p-value for the overall partial F-test is accompanied by a fairly low power. This indicates that if in fact a difference would exist, the chosen experimental design would not be able to detect it most of the time. In such instances, an application of this new approach may be of use in providing appropriate analytical methodology. The application of techniques that lend themselves to the analysis of individual differences in animals will increase appreciation for how these differences may be elucidated at a molecular and biochemical level of an organism.

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| Table 1. Comparison of Regression Results between the Two Models throughout Different Brain Sections. | | | | | | | |
|---|---|---|---|---|---|---|---|
| # | Section | Model 1 | | | Model 2 | | |
| | | $\%CV_{max}$ | %RMS | $R^2$ | $\%CV_{max}$ | %RMS | $R^2$ |
| 1 | hypo | 9 | 6.1 | 0.81 | 42 | 5.8 | 0.94 |
| 2 | hippo | 12 | 8.7 | 0.92 | 62 | 8.0 | 0.94 |
| 3 | piri | 7 | 8.7 | 0.93 | 16 | 7.7 | 0.96 |
| 4 | lc | 10 | 8.9 | 0.81 | 37 | 9.4 | 0.82 |
| 5 | fctx | 5 | 6.7 | 0.95 | 11 | 6.3 | 0.96 |
| 6 | cere | 9 | 13.3 | 0.83 | 34 | 12.9 | 0.86 |

*Model 1 = restricted Model (8 parameter) ; Model 2 = full model (14 parameters); $\%CV_{max}$ = maximum value coefficient of variation as calculated according to Eq. 7; %RMS = root mean square in percent as calculated using Eq. 9; $R^2$ = coefficient of determination as computed using Eq. 10*

*Abbreviations: 1. hypo = hypothalamus; 2. hippo = hippocampus; 3. piri = piriformis; 4. lc = locus coeruleus; 5. fctx = frontal cortex; 6. cere = cerebellum.*

*For brain sections # 1-6 the maximum correlation coefficient between the parameter estimates of model 1 was -0.40 and -0.76 for Model 2.*

**Table 2. Results of Significance Tests for Inter-Individual Variability in Different Brain Sections of Rats.**

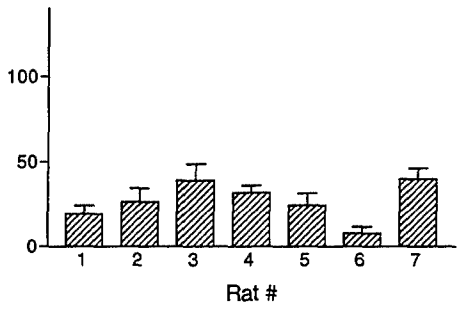
| # | Section | $SSE_1$<br>$SSE_2$ | $df_1$<br>$df_2$ | $F(df_1\text{-}df_2,df_2)$ | p | Power<br>$\alpha = 0.05$ |
|---|---|---|---|---|---|---|
| 1 | Hypo | 1.394 | 40 | 1.758 | 0.138 | 0.56 |
| | | 1.064 | 34 | | | |
| 2 | Hippo | 2.801 | 41 | 2.255 | 0.061 | 0.70 |
| | | 2.020 | 35 | | | |
| 3 | Piri | 5.473 | 41 | 2.823 | 0.024* | 0.82 |
| | | 3.688 | 35 | | | |
| 4 | Lc | 2.073 | 41 | 0.246 | 0.958 | 0.11 |
| | | 1.789 | 35 | | | |
| 5 | Fctx | 3.703 | 41 | 1.947 | 0.100 | 0.64 |
| | | 2.777 | 35 | | | |
| 6 | Cere | 10.782 | 41 | 1.441 | 0.227 | 0.46 |
| | | 8.647 | 35 | | | |

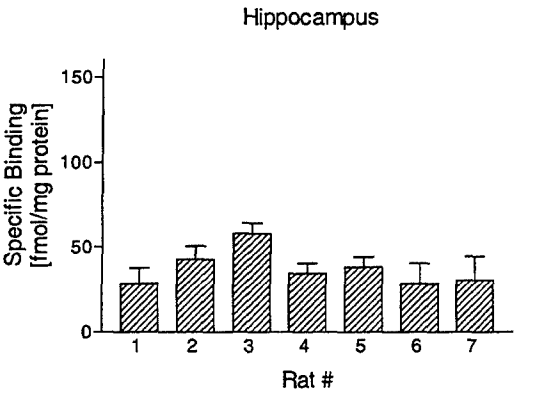
*$SSE_1$ = sum of squared error of Model 1 (reduced model) at the minimum; $SSE_2$ = sum of squared error of Model 2 (full model) at the minimum; $df_1$ and $df_2$ are the respective degrees of freedom. The F-ratio is revealed according to Eq. 4. Statistical power expressed as fraction is empirically determined with 2000 independent Monte-Carlo simulations ($\alpha$ two-sided). Abbreviations: 1. hypo = hypothalamus, 2. hippo = hippocampus, 3. piri = piriformis, 4. lc = locus coeruleus, 5. fctx = frontal cortex, 6. cere = cerebellum.*

*$* p \leq 0.05$*

| Table 3. Results of a modified Scheffe-Test for Inter-Individual Variability in Area Piriformis in Brains of Different Rats. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *RAT #* | | *1* | *2* | *3* | *4* | *5* | *6* | *7* |
| | *MEAN* | *122.9* | *100.9* | *134.6* | *130.5* | *117.2* | *75.9* | *98.8* |
| *1* | *122.9* | 1 | 0.95 | 0.99 | 1.00 | 1.00 | 0.33 | 0.92 |
| *2* | *100.9* | 0.95 | 1 | 0.72 | 0.82 | 0.99 | 0.91 | 1.00 |
| *3* | *134.6* | 0.99 | 0.72 | 1 | 1.00 | 0.98 | 0.11 | 0.65 |
| *4* | *130.5* | 1.00 | 0.82 | 1.00 | 1 | 1.00 | 0.17 | 0.77 |
| *5* | *117.2* | 1.00 | 0.99 | 0.98 | 1.00 | 1 | 0.49 | 0.98 |
| *6* | *75.9* | 0.33 | 0.91 | 0.11 | 0.17 | 0.49 | 1 | 0.94 |
| *7* | *98.8* | 0.92 | 1.00 | 0.65 | 0.77 | 0.98 | 0.94 | 1 |

*Multiple comparison results using a modified Scheffe-test comparing animals after the overall F-test revealed a probability $p \leq 0.05$ (cf. Table 2). The F-ratio was calculated according to Eq. 4. The shaded area provides information about a certain rat with its estimated specific binding (mean value), expressed in fmol/mg protein. The white area shows the computed probability of testing individual differences (c.f. Eq. 11).*


100
50
0
1 2 3 4 5 6 7
Rat #


Hippocampus
Specific Binding
[fmol/mg protein]
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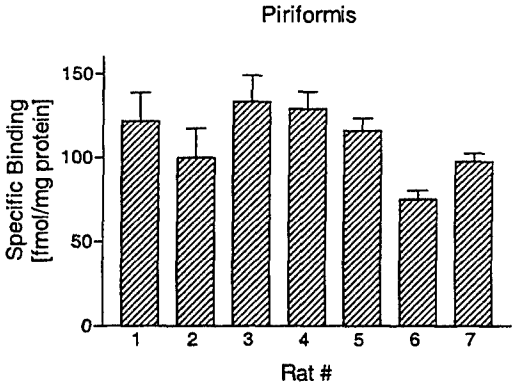
100
50
0
1 2 3 4 5 6 7
Rat #


Piriformis
Specific Binding
[fmol/mg protein]
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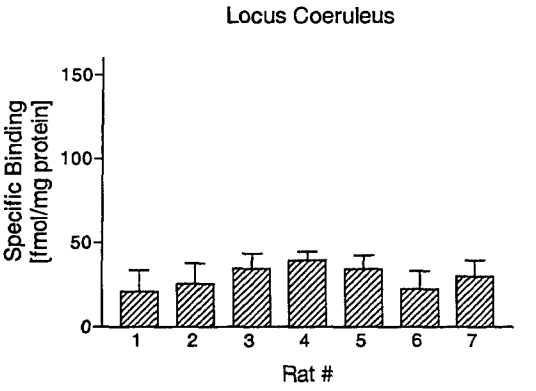
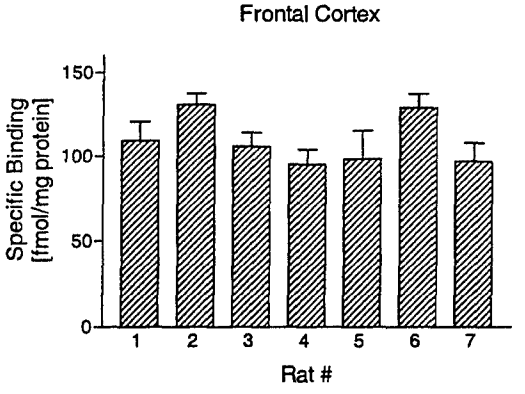
100
50
0
1 2 3 4 5 6 7
Rat #


Locus Coeruleus
Specific Binding
[fmol/mg protein]
150
100
50
0
1 2 3 4 5 6 7
Rat #


Frontal Cortex
Specific Binding
[fmol/mg protein]
150
100
50
0
1 2 3 4 5 6 7
Rat #

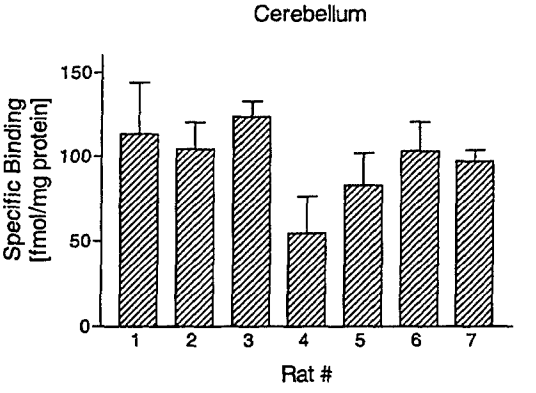

Cerebellum
Specific Binding
[fmol/mg protein]
150
100
50
0
1 2 3 4 5 6 7
Rat #

Figure 1. Individual levels of specific CRH-receptor sites for [$^{125}$I]-Tyr$^{0}$ oCRH in rat brain hypothalamus, hippocampus, piriformis, locus coeruleus, frontal cortex and cerebellum after repeated treatment with i.c.v. oCRH, expressed as mean and SEM. Membrane-suspensions of each section were incubated for 2 h at room temperature in the presence of 0.1 nM [$^{125}$I]-Tyr$^{0}$ oCRH and 5.4 nM unlabeled oCRH (mean protein content ranged from 0.1 to 0.3 mg protein/ml assay). Abscissa: seven individual rats. Ordinate: specific binding in fmol/mg protein as revealed from equation 1. SEM was calculated according to Eq. 2.